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Identifying, Developing and Releasing Insect Biocontrol Agents for the Management of *Phragmites australis*

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ABSTRACT: Introduced *Phragmites australis* is rapidly spreading in North America, particularly along both East and West Coasts, but increasingly in the Midwest as well. Initially from Europe, the introduced genotype is rapidly replacing diverse native wetland plant communities and their associated biota with near monocultures. Furthermore, spread of the European genotype is threatening endemic native genotypes (now identified as a subspecies *Phragmites australis americanus*), with few remaining populations in the East while Midwestern and Western populations are more common. A lack of successful long-term control techniques resulted in the initiation of a project in 1998 that was aimed at developing biological control of the introduced genotypes. The current report details research accomplishments in host specificity testing, ecological impacts, hybridization potential, and long-term monitoring from June 2011 to May 2012.

INTRODUCTION: The status of *Phragmites australis* (common reed) as native or introduced and questions surrounding its invasiveness in North America had puzzled researchers for decades (Marks et al. 1994, Tewksbury et al. 2002) until the present day existence of both native North American and introduced European haplotypes was confirmed through genetic methods (Saltonstall 2002). Initial introduction of European material occurred sometime in the early part of the 19th century, probably at Atlantic coast ports, and all introduced populations examined in North America belong to the same haplotype (M), which is the most widespread haplotype worldwide. While native genotypes appear more abundant in the Midwest and the Southwest, Type M has nearly entirely replaced native genotypes in the Northeast and Mid-Atlantic regions of the US (Saltonstall 2002). These native populations may be declining (Marks et al. 1994), potentially accelerated by local introduction of nonindigenous genotypes (Marks et al. 1994, Blossey 2003a). The native genotypes have recently been recognized as a distinct subspecies *Phragmites australis americanus* Saltonstall, P.M. Peterson and Soreng (Saltonstall et al. 2004). Hybridization between native and introduced populations has been experimentally achieved in the lab (Meyerson et al. 2008) and there is now preliminary evidence for hybridization between North American and European genotypes (Saltonstall and Blossey, unpublished data).

The rapid expansion of introduced *P. australis* populations in both freshwater and brackish North American wetlands and the resulting ecological impacts are generally (but not always) considered detrimental (Marks et al. 1994, Chambers et al. 1999, Meyerson et al. 2000, Rooth and Stevenson 2000). Expanding populations threaten ecological, agricultural, recreational and other ecosystem functions and interest in controlling *P. australis* in urban, rural, agricultural and natural areas in the Great Lakes region remains strong and is increasing in many other areas. The entire arsenal of

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14. ABSTRACT

Introduced Phragmites australis is rapidly spreading in North America, particularly along both East and West Coasts, but increasingly in the Midwest as well. Initially from Europe the introduced genotype is rapidly replacing diverse native wetland plant communities and their associated biota with near monocultures. Furthermore, spread of the European genotype is threatening endemic native genotypes (now identified as a subspecies Phragmites australis americanus), with few remaining populations in the East while Midwestern and Western populations are more common. A lack of successful long-term control techniques resulted in the initiation of a project in 1998 that was aimed at developing biological control of the introduced genotypes. The current report details research accomplishments in host specificity testing ecological impacts, hybridization potential, and long-term monitoring from June 2011 to May 2012.

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control methods available to land managers — including herbicides, mowing, disking, dredging, flooding, draining, burning, covering and grazing — has been tried to manage *P. australis* (Marks et al. 1994). Permanent control may be achieved in areas where tidal flushing with full strength saltwater can be achieved, but this would be restricted to previously diked coastal marshes. Currently, the most widespread and successful control method appears application of glyphosate (or other herbicide) late in the growing season, followed by prescribed burning or mechanical removal of dead stalks, and often subsequent application of herbicide the next year (Blossey and McCauley 2000, Ailstock et al. 2001). In order to maintain areas with low *P. australis* abundance, however, retreatments are usually necessary every 3-5 years, representing a continued strain on management budgets. In addition, negative side effects on non-target plants are inevitable if non-selective herbicides are used over large areas. The inability to achieve long-term control of invasive *P. australis* resulted in the initiation of a biocontrol program that has — since 1998 — researched possibilities to use natural enemies from the native range as control agents.

In the past 3 years, the program targeting introduced *Phragmites* has focused on several promising European potential biological control agents, with large impacts on *P. australi*s growth and performance. Host specificity testing was conducted in a Rhode Island quarantine facility (Richard Casagrande, PI and Lisa Tewksbury), while additional host specificity work and maintenance of the appropriate insect rearing colony was maintained in Europe at CABI Switzerland (Patrick Häfliger and Hariet Hinz). In addition to this work, the authors conducted surveys for potential soil pathogens influencing *Phragmites* success (Eric Nelson and Ellen Crocker); continued investigations on the impact of different *Phragmites* populations on native fauna and flora; and conducted an economic and ecological assessment of *Phragmites* invasion and management (Bernd Blossey, Jeremy Dietrich, Laura Martin and Jillian Cohen). Many experiments are ongoing, and much of this information will need to be summarized and published in the near future. The purpose of the current funding was to enhance the biocontrol program and had the following five major objectives:

- **Objective 1:** Identify potential agents for more in-depth study
- **Objective 2:** Develop testing procedures and conditions for host-specificity studies and collect data on host specificity of identified agents
- **Objective 3:** Develop laboratory/greenhouse mass-rearing procedures
- **Objective 4:** Assist with selection of prerelease sites for long-term monitoring
- **Objective 5**: Assess the extent of hybridization between native and introduced genotypes

This work is jointly performed by a number of investigators with Bernd Blossey as the lead PI and collaborators at Cornell University (Eric Nelson, Plant Pathology; and several graduate students), University of Rhode Island (Richard Casagrande and Lisa Tewksbury) and overseas work at CABI Switzerland (Hariet Hinz and Patrick Häfliger).

Accomplishments during the reporting period. The previous report from May 2011 detailed accomplishments for Objective 1 and this information will not be repeated here. Similarly, the authors outlined much of the details for host specificity testing (Objective 2) in the previous report and will only briefly mention previous procedures as needed to understand the technical details. This technical note will focus on the accomplishments during the reporting period and the

remaining work program. At the time of report writing, the field season has just started; thus, much of this year's host specificity work program is still in progress. The team will try to elaborate as much as possible on ongoing work.

Objective 2: Develop testing procedures and conditions for host-specificity studies and collect data on host specificity of identified agents

Implementation of a biological control program targeting invasive *P. australis* in North America will encounter a set of unique challenges, and much of this information (including a full list of host specificity non-target species and their selection criteria) was detailed in the May 2011 report and will not be repeated here. But in particular, the existence of native endemic North American genotypes of *P. australis americanus* (Saltonstall 2002, Blossey 2003a, b, Saltonstall 2003) requires extra scrutiny.

Following guidelines by the Technical Advisory Group for Biological Control Agents for Weeds (TAG), the authors developed a tentative list of approximately 45 plants for host range testing. The basis of the experimental procedures is plant collections in Europe at CABI Bioscience Centre in Delémont, Switzerland and at URI in Kingston, RI. Tests in Europe will allow for studying the behavior of control agents in common gardens, in field cages or under unconfined conditions, and many of these tests are either in preparation or ongoing. Work at URI, where an approved quarantine facility is available, is focusing on testing native North American species. At both locations, test plants are being maintained and propagated in common gardens.

All potential control agents are shoot-boring moths in the genera *Archanara* (*A. geminipuncta, A. neurica, and A. dissoluta* and *Arenostola phragmitidis*. All species are univoltine (one generation/year); they overwinter as eggs on dead stems and first instar larvae emerge when early shoot growth of *P. australis* begins in early spring (between April and June depending on latitude and local climate conditions). The tests were conducted both in Europe at CABI Bioscience Center in Switzerland as well as under quarantine conditions at the University of Rhode Island. In both venues, the team relied on a mass production operation by Patrick Häfliger at CABI to provide eggs for shipments to RI since it was not possible to rear these insects under quarantine conditions. This mass production is quite labor intensive as it requires rearing individual larvae in cut *Phragmites* stem pieces, frequently transferring larva by hand, and collecting eggs after moth oviposition in cages. While all moth species can be reared with enormous efforts (significant student and summer help is required to maintain these colonies), two species (*Archanara geminipuncta* and *A. neurica*) proved most amenable to these procedures and therefore, increasing colony size would be possible. Much of the work accomplished involves the two most widespread and abundant European species that also proved most amenable for mass production. The other species is maintained and the authors will focus on these as the work for the first two species is nearing completion (pending availability of funding).

Throughout the host specificity testing, the authors have relied largely on tests using first instars and either potted plants or stem sections. In addition, adult choice experiments have been conducted in Europe using either field cages or open releases exposing different introduced and native *P. australis* genotypes to select potential control agents for oviposition (see below). The specific larval requirements demand close synchronization of shoot growth and initial larval feeding, including on the host species, *P. australis,* to obtain valid controls. Even the difference

of a few days renders host tissue unacceptable to first instars of either moth species. While this requirement is a great help in rendering the vast majority of test plant species unacceptable to the potential biocontrol agents, this has made the work extremely challenging as it requires rearing of plant species with very different phenologies to initiate shoot growth at the correct time. Delaying egg hatch, while an appealing possibility due to effective and easy storage of eggs in a refrigerator, appears to limit the ability of later hatching larvae to successfully attack test plants and even shoots of the original host despite apparently being of the right phenological stage. Such problems are not uncommon in biocontrol programs, however, the extent of the tissue specificity and synchronization that is required is quite extraordinary.

Quarantine testing. Host specificity testing is conducted in various stages or venues. The reason for different testing scenarios is that they all have various levels of resembling a field situation with the most reductionist (no-choice larval transfer) often delivering false positives (indicating host acceptance while in the field this would never happen). Consequently, host specificity screening procedures use increasing levels of realism (which usually increases the costs and requires work in the native range for field tests) for species that have been accepted in reductionist experiments moving from test plant tissues to potted plants, to the field. Host specificity screening procedures also use increasing levels of complexity from no-choice to single-choice, to multiple-choice and from caged tests to open field tests. Overall, the authors have conducted host specificity tests in quarantine (for tests in Europe see the next section) as:

1. First instar larval transfer tests (no choice, or stage 1)
2. Larval transfer and development test (no choice, or stage 2)

The first test exposes first instar larvae to host plants and one replicate consists of one larva exposed to one to four stems enclosed inside a 5 cm diameter acrylic tube that is either 30.5 cm or 46 cm tall. Larvae are allowed 5 days to enter stems and feed before each replicate is evaluated. All stems are dissected, and any stem attack is recorded and photographed as represented by feeding damage, frass, or entrance and exit holes. In this test sequence, the authors are now able to achieve sustained testing method success with successful larval feeding in the *Phragmites* controls: 93% feeding of replicates with *A. geminipuncta*, and 77% of larvae still alive after the 5-day trial.

At the end of the 2011 season, all but two test plant species had been tested for *A. geminipuncta* and 10 for *A. neurica* in stage 1 testing. The remaining two species were completed for *A. geminipuncta* in spring 2012, but there are three species to test for *A. neurica* (Table 1). All of the remaining plant species are available for testing. Problems with longevity of cut stems in this test and lack of *A. neurica* eggs prevented the team from completing this testing sequence in 2012. The authors will reduce the exposure time of stems by 2 days in 2013 (assuming sufficient eggs are obtained from CABI) allowing for assessment of larval feeding before stems begin to decay.

Test results for this set-up demonstrate that the risk for attack on native *Phragmites* cannot be excluded, although larval performance and survival is clearly enhanced on introduced genotypes (data not shown). There is minor attack on other plant species under quarantine conditions, but these are most likely lab artifacts, since such attack is never reported in the literature and larval survival is greatly reduced (data not shown). There are also several plant species for which the same tests were conducted both in Europe and North America. The authors have indicated discrepancies between the results from Europe (Table 2) and those obtained in quarantine in

Table 1. Summary of no choice, first instar larval transfer host specificity test results for *A. geminipuncta* and *A. neurica* in quarantine. (+ = some feeding damage; X = no feeding damage ([X] indicates test done at CABI), O for not yet tested) * indicates discrepancies between this test and those initial test results conducted in Europe at CABI using similar techniques (Table 2). Discrepancies suggest the artificiality of this test.

Species Tested	*A. geminipuncta*	*A. neurica*
Phragmites australis (introduced)	+	+
Phragmites australis (native)-ME	+	+
Phragmites australis (native)- CA	+	+
Phragmites australis (native)-NY	+	+
Agropyron cristatum	X	[X]
Andropogon gerardii	X	X
Aristida purpurea	X	X
Arundinaria gigantean subsp. tecta	+	X
Arundo donax	+*	X
Avena sativa	X	X
Carex lurida	X	X
Cortaderia selloana	+	+
Cyperus haspan	X	X
Dactylis glomerata	X	X
Danthonia spicata	X	O
Distichlis spicata	X	X
Elymus virginicus	X	X
Eragrostis trichodes	X	[X]
Glyceria striata	X	X
Hordeum vulgare	X	O
Iris versicolor	X	+
Juncus effusus	X	X
Leersia oryzoides	X	X
Lolium perenne	X	[X]
Muhlenbergia racemosa	X	X
Oryza sativa	+	X
Panicum virgatum	X	X
Phalaris arundinacea	+	+
Pontederia cordata	X	X
Saccharum officinarum	X	+
Schoenoplectus acutus	X	+
Schoenoplectus americanus	+	X
Secale cereale	X	O
Setaria italica	X	X
Sorghastrum nutans	X	X
Sorghum bicolor	X	X
Sparganium americanum	X	X
Spartina alterniflora	+	+
Spartina cynosuroides	+	X
Tripsacum dactyloides	X	X
Triticum aestivum	+*	X
Typha angustifolia	X	X
Typha latifolia	X	X
Zea mays	X	X
Zizania aquatic	+	+
Zizania palustris	X	X
Zizaniopsis miliacea	X	X

Table 2. Results of no-choice larval development tests with *Archanara geminipuncta* and *A. neurica* conducted at CABI in Europe (Data are means ± SE) N/A indicates this species was not tested for this control agent.

	A. geminipuncta			*A. neurica*		
Test plant species	**N**	**# stems attacked**	**# live larvae**	**N**	**# stems attacked**	**# live larvae**
Phragmites australis	5	2.8±0.4	4±0.3	5	2.8±0.4	2.2±0.4
Phalaris arundinacea	5	0	0	5	0	0
Typha sp. old	5	0	0	5	0	0
Typha latifolium young	5	0	0		N/A	N/A
Eragrostis trichodes	5	0	0	5	0	0
Schoenoplectus acutus	5	0	0	5	1.0	0.6±0.6
Schoenoplectus americanus	5	0	0		N/A	N/A
Lolium perenne	5	0	0		N/A	N/A
Spartina cynosuroides	3	0.7±0.3	1±0.6	5	0.2±0.2	0
Agropyron cristatum		N/A	N/A	5	0	0
Iris versicolor		N/A	N/A	5	0	0
Glyceria striata	5	0	0		N/A	N/A
Setaria italica	5	0	0		N/A	N/A
Zizania aquatica	5	0.2±0.2	0		N/A	N/A

Table 1 using an asterisk (*). In all instances, test results in Europe demonstrate the inability of larvae to attack these plant species. The reasons for these differences are unknown, but the results highlight the artificiality of these experiments and the need for more sophisticated tests. As a next step in quarantine, all species attacked in stage one tests are moved to stage two, no choice, larva development tests.

Larval development tests conducted over the past seasons (and currently ongoing) use the plant species attacked in no-choice larval transfer tests to further assess their suitability for providing useful substrate to continue larval development, with native and introduced *Phragmites* as controls. The authors prepare flats (46 x 46 cm, 15cm deep) to grow each of the species that were attacked in the first stage testing by one of the insects being tested. The flats were prepared in the fall/winter and were kept at 10°C for at least three months. At the appropriate time, the team transferred flats into the greenhouse to initiate shoot growth and then into quarantine where larval transfers were conducted. The authors conduct 5 replicates for each test plant species and 5 larvae per replicate were transferred. The number of stems (which varies among replicates) were counted and after 10-14 days, the authors harvest stems and dissect them to look for feeding marks and the presence of live larvae. Every time a test run is performed, introduced *Phragmites* flats are used as a control. Data is reported as the total number of stems exposed, the total number of stems attacked, and the total number of larvae alive (Table 3); neither means nor reporting of individual replicates appears appropriate.

In the second stage testing of *A. geminipuncta*, the authors had good stem penetration and internal feeding on the *Phragmites* controls (12/25 and 23/25 larvae fed within stems), but the effort of rearing larvae for ten days was not as successful. The team had hoped that the *A. geminipuncta* larvae would continue to develop within the flat, moving to a new stem and that live larvae would be found after 10 days. After 10 days, only 12/49 stems (25%) had internal feeding, with no live larvae in the first trial, and 23/50 stems (46%) were fed upon, with only 4 live larvae in the second trial (Table 3). Consequently, this second-stage testing is best considered as a second round of stage one testing with

increased replication, and it further shows the extreme specificity of the larvae requiring stems of a certain age and of a certain diameter. Achieving large enough stem diameters in pots or flat grown plants in the greenhouse or quarantine is a challenge that has proven difficult to overcome. The authors are considering moving some testing of critical test plant species to Europe for oviposition and larval development tests. In Europe, these tests can be conducted in the field or a common garden with open grown plants; this will possibly achieve more reliable results.

Table 3. Second Stage (no choice, larval development) test results for *A. geminipuncta and A. neurica*. Tests for *A. neurica* are ongoing or will be completed in 2013. Data reflect a summary of 5 replicates/test plant species/insect. Listed are the total number of stems exposed to a total of 25 larvae; the number of stems with signs of larval attack; and the number of live larvae after 14 days of exposure.

	A. geminipuncta			*A. neurica*		
Test plant species	**No. of stems**	**No. of stems w. feeding**	**No. of live larvae**	**No. of stems**	**No. of stems w. feeding**	**No. live larvae**
Phragmites australis (exotic)	49	12	0			
Phragmites australis (exotic)	50	23	4			
Arundinaria gigantea tecta						
Arundo donax	76	6	0			
Cortaderia selloana						
Oryza sativa						
Phalaris arundinacea	127	0	0			
Saccharum officinarum						
Schoenoplectus acutus						
Schoenoplectus americanus	117	0	0			
Spartina alterniflora	119	5	1			
Spartina cynosuroides	95	0	0			
Triticum aestivum						
Zizania aquatica	57	0	0			

Of the six plants tested for A. *geminipuncta*, positive feeding was found on *Arundo donax*, and *Spartina alterniflora* (Table 3). The other four plants (*Phalaris arundinacea*, *Schoenoplectus americanus*, *Spartina cynosuroides*, and *Zizania aquatica*) can probably be ruled out as potential hosts at this point based upon the negative results with large sample sizes. (The previous year's data will be revisited; that data led to stage two testing and the reclassifying of results, whereby external feeding was present but no feeding within stems).

Fortunately, the authors also have assessment data from previous seasons in Europe concerning the possibility of larval development in test plant species that previously showed attack using first instar larval transfer tests (either in tests conducted in Europe or at URI). In Europe, the possibility existed to use both *A. geminipuncta* and *A. neurica,* since sufficient larvae were available to run these tests. While these larval transfer tests clearly "weed out" an additional number of species, (Table 2) two plants are seen (*Schoenoplectus acutus* and *Spartina cynosuroides*) that allow the occasional larvae to continue development, although in all instances, these larvae remain much smaller than those feeding and developing in *P. australis*. Interestingly, different results can be obtained in different years, indicating that the artificiality of these tests (no choice) can affect outcomes. But it is usually only a single larva that continues to survive. At present, the authors

have not attempted to rear these larvae through to pupation and adult stage. It is doubtful that larvae of these two species would complete development, but further tests are needed.

Work in Europe at CABI. The work of European collaborators Hariet Hinz and Patrick Häfliger is currently focusing on maintaining rearing colonies of potential biocontrol agents, shipments of eggs, and field testing of endemic *P. australis americanus* genotypes in the native range of the authors' control agents. Work during the past season has focused on developing and testing a novel release and host specificity screening method, using open field testing and assessing oviposition behavior of adult moths.

In biocontrol programs worldwide, it has been shown that field tests are more reliable and predictive. Based on the evidence from hundreds of programs targeting other plant species, the most encouraging and promising data for the possibility of developing biological control for *P. australis* in North America come from open field tests conducted last summer in Switzerland. The authors exposed potted native North American *P. australis americanus*, introduced North American genotypes, and European genotypes of *P. australis* to gravid ovipositing females of *A. geminipuncta* at two different common gardens in Switzerland. This design (Fig. 1) was chosen to test oviposition behavior of *A. geminipuncta* in the contact zone of native and introduced *P. australis* under conditions that were as natural as possible. The original plan was to carry out an open-field oviposition test with potted plants set up at the border of a field site. However, an initial preliminary test in 2010 showed that stems on potted plants were not used for oviposition in the presence of stems grown in the field, so the authors developed this new approach.

In early July, the team created four different patches in two gardens in Delémont, Switzerland. Patch 1 contained 7 pots of native and 7 pots of introduced North American *P. australis*; patch 2 contained 14 pots of native *P. australis americanus*; patch 3 contained 14 pots of introduced North American *P. australis*; and patch 4 contained 14 pots of European *P. australis* (Figure 1). Pots were placed close to each other with pot rims touching. Within origin, three different populations were used and randomly mixed. In mid-July, eight mated *A. geminipuncta* females were released in the center of this setup. At a second site, females were released in the center of a patch. All females were marked with fluorescent powder (Figure 2) to allow the authors to follow dispersal between patches. In addition, eggs laid by different color-coded females can be easily distinguished since remnants of the fluorescent powder identify the female release source. Two weeks after release of moths, all stems were harvested and searched for eggs.

The authors have evaluated data from one of the two common gardens for this report. When females were released in the center of each of four patches and marked with fluorescent powder, eggs were found only on European genotypes and introduced North American *P. australis;*none were found on native North American genotypes. In fact, females moved among patches and did not remain in their original release locations. Females moved from native *P. australis americanus* to introduced or European genotypes for oviposition, although the number of eggs and egg clusters located was small. This is thus far the strongest evidence for selective behavior of ovipositing females. While the number of females released and ovipositions recorded is small, the authors' experimental design appears sophisticated and realistic enough.

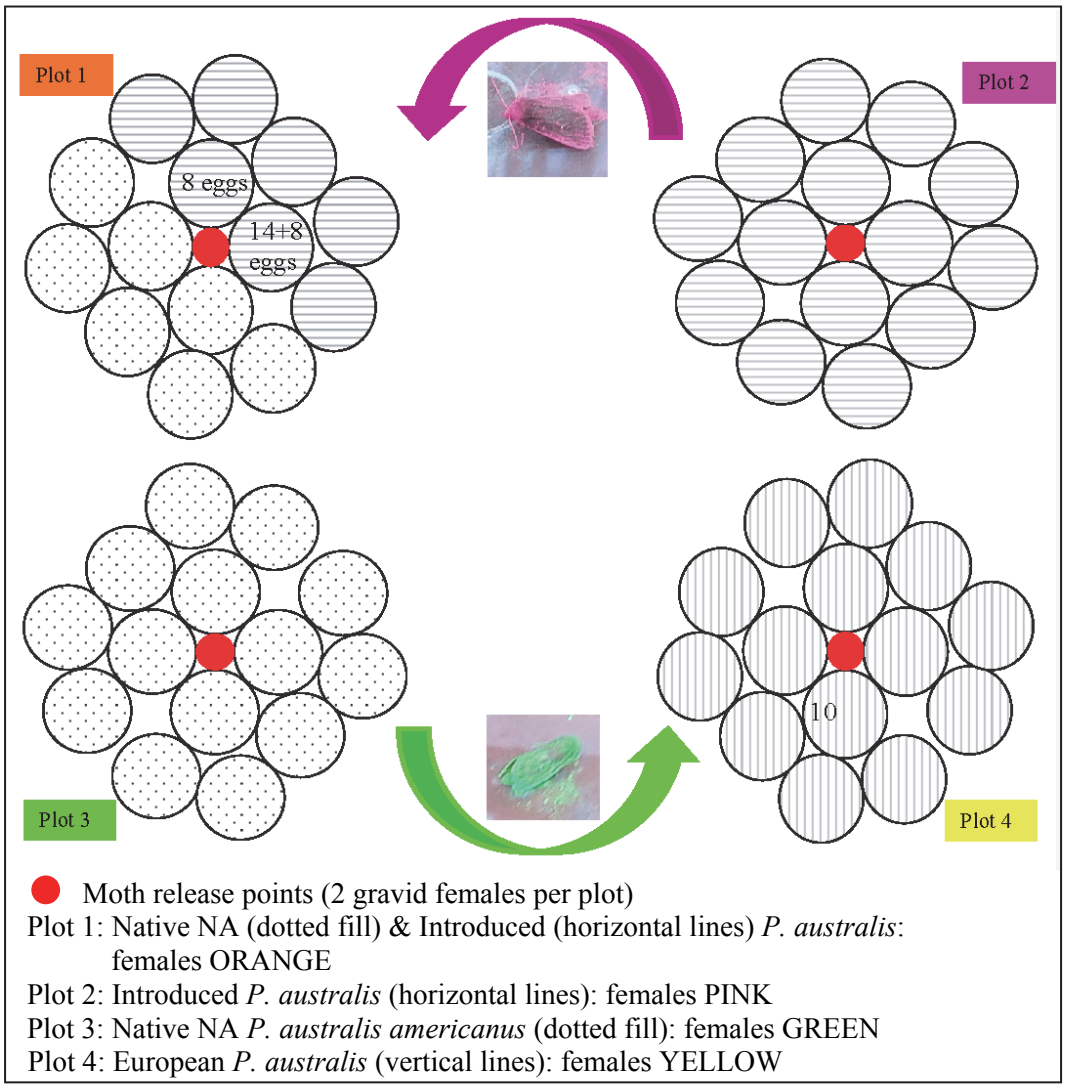

Figure 1. Arrangement of the four different plots (each with 14 potted plants = circles) of the open-field oviposition test with *A. geminipuncta* in a common garden. Origin of *P. australis* clones offered as potted plants is coded using dots or lines) and females were color coded using fluorescent dye (Fig. 2). Numbers within circles represent the number of eggs laid on a particular plant. In one case (14/8), two different egg batches were found. Dust of eggs allows researchers to determine females' origin. Color-coded sweeping arrows represent dispersal of moths from one patch to the other.

The research team also evaluated whether a release in the middle of the 4 plots array would create valuable data. However, all females dispersed and no eggs were found on any Phragmites stems, making this an invalid test. More plants are currently being propagated along with additional insects and with the approved increase in the overall budget; these experiments will be repeated with more adults and more species in 2013. The 2011 data are extremely promising. There is a strong desire to safeguard native North American *P. australis americanus* genotypes, and lack of oviposition is certainly a major safety factor if this can be confirmed in the future.

Figure 2. Adult *A. geminipuncta* females marked with different fluorescent powder immediately before release into *P. australis* experimental patches.

Objective 3: Develop laboratory/greenhouse mass-rearing procedures

In all weed biocontrol programs, control agents are initially in very short supply during initial host specificity testing, or even once field release permits are obtained. Traditionally, releases were made into field sites that were intended to serve as future "nursery sites;" i.e., sites where collection of control agents was made after populations increased sufficiently. This approach is problematic for various reasons, including the potential long wait for control agents to build high populations, the danger of making poor choices for field release sites, and the loss of "momentum" in a control program. The purple loosestrife control program has been an interesting counterexample. The very first introductions were spread across the continent and included many different collaborators in different climate zones and involved experiments to learn about the best release procedures (Hight et al. 1995). Within a few years after the initial release, mass production procedures had been developed; some of these procedures were so simple that high school students were able to perform them (Blossey and Hunt 1999, Blossey et al. 2000). Written and videographic instructions for performing the mass production were also distributed.

Various approaches have been attempted by the research team using semi-artificial media that was successfully used to rear Lepidoptera (at URI) and weevils (at Cornell). The team's experience suggested that it would be reasonably easy to adopt these procedures for stem mining *Archanara* larvae and thus maintaining year-round colonies. A sufficient supply for host specificity testing and for subsequent field releases in North America could also be maintained, should these moths be approved as biocontrol agents.

The team began experimenting with approaches to rear two of the stem-boring species, *A. geminipuncta* and *A. neurica,* on an artificial diet to replace labor-intensive methods Patrick Häfliger developed in Switzerland (with larvae reared on cut *P. australis* shoots or potted plants). The initial diet was a high wheat-germ diet (a gypsy-moth wheat germ diet with premixed agar

from MP Biomedicals, Inc.) with ground-up fresh *Phragmites* stems added to the diet (12% of the total dry weight). Initially, the authors placed the diet in 1 or 2 oz. cups, and replaced food after two weeks. In neither case did the larvae successfully develop to adult. With both species, over 50% of larvae-fed diet died within the first two weeks. These disappointing results resemble results obtained during some preliminary attempts in Switzerland where artificial diet rearings did not result in an increase in the colony. Larvae failed to complete development on artificial diet, even if they initially fed on the semi-artificial diet produced in the present study.

Consequently, the team attempted to change procedures to improve the rearing of stem miners, assuming that internally feeding larvae require hollow tubes with food lining the walls for a successful development. That most larvae died in the early instars is an indication that larvae did not accept the food offered and the team hoped to improve on the procedures. A diet was placed either in 1 oz. cups or 6 mm diameter plastic drinking straws, and food was replaced after 5-7 days. Neonates were placed individually in cups or straws with cotton caps. The drinking straws were chosen to resemble the effect of feeding inside a stem. The team placed >50 *A. neurica* and >50 *A. geminipuncta* on diet cups or into drinking straws and monitored weekly survival for 6 weeks. While slight improvements in the ability to keep larvae alive were noted, as compared to earlier results, no larvae of either species was able to complete development to the pupal stage on the artificial diet. In fact, the vast majority of mortality again occurred in the first two weeks.

After two years of rearing attempts results under this contract, the authors abandoned intensive efforts to develop mass rearing techniques based on an artificial diet formulation. Initially, the lack of success created a rush to develop even more variations of artificial diet formulation but none of these attempts resulted in successful larval development. Instead, more energy and time was spent on this diet development than was initially anticipated. The team is uncertain whether it is diet quality, rearing conditions — or a combination of both — that is causing the failure. Due to the restrictions based on the approaches (requiring work in quarantine) used, the team is unable to assess problems in any meaningful experimental way. Further diet development has been stopped, after two years of unsuccessful attempts, preventing the further "wasting" of a lot of the available larvae and financial and work force resources. While not entirely satisfactory and labor intensive, the rearing of individual larvae of stem cuttings can provide sufficient eggs for host specificity testing, investing in more student or technical help will be necessary to maintain larger colonies if rearing demands and field releases are envisioned in the future; particularly if this work is anticipated to occur in quarantine in North America. Field-based mass production techniques should also be considered, most likely in Europe, where Patrick Häfliger is successful in continuing to produce large quantities of eggs; although he is experiencing some problems for some species as well after 10 years of keeping colonies for testing and shipment of eggs in Europe. Fortunately, there are known field locations where it is possible to collect larvae for rearing purposes, should colonies fail at some point in the future.

Objective 4: Assist with selection of pre-release sites for long-term monitoring

In collaboration with land managers, the authors have selected a number of *Phragmites* sites as long-term study sites in RI and NY (N=14 paired). Currently, these sites serve initially as monitoring sites to assess *Phragmites* expansion rates (native and introduced) until control agents are available; then the sites serve as release sites. Paired sites are located in similar habitat types and in the same geographic region (See Figure 3, Table 4 for NY sites). One of the two

sites will serve as a future insect release site, while the other functions as a control (at least initially until insects disperse widely on their own). Sites are located sufficiently distant from each other (5-10km) to prevent immediate dispersal of control agents among sites. Once control agents have reached higher abundances, release sites can also serve as collection sites for re-distribution of control agents.

Figure 3. Long-term monitoring sites in NY State.

Table 4. Locations and descriptions of *P. australis* sites in New York.

Site	Location	Latitude	Longitude	*Phragmites* Origin
Carncross	Montezuma	43°04′56.624′′	76°42′38.241′′	Native
Martens Marsh	Montezuma	43°05′04.784′′	76°42′33.583′′	Introduced
Eagle Point	Butler	43°10′16.123′′	76°47′34.704′′	Introduced
Eagle Point	Butler	43°10′16.123′′	76°47′34.704′′	Native
Bear Swamp	Moravia	42° 44' 25.645"	76°17.511′	Native
Lakeview WMA	Pulaski	43°45′06.148′′	76°11′55.568′′	Native
Colwell Pond	Ellisburg	43°41′57.72′′	76°11′37.43′′	Introduced
Rochester 531	Syracuse	43°10′30.271′′	77°46′06.462′′	Introduced
Syracuse 481	Syracuse	43°09′27.847′′	76°08′53.047′′	Introduced
Utica 5S	Utica	43° 1' 8.965"	75.028918	Introduced
Utica 790 E	Utica	43°06′55.540′′	75°14′03.292′′	Introduced

From August – September 2009, the authors established 15-20 permanent $1m^2$ quadrats along multiple transects through each *P. australis* clone that spans the "invasion front" of native or non-native clones. The number of quadrats depends on the extent of the clone at each site. This allows for an expansion rate assessment for each clone (if any) and a rate of spread comparison between native and introduced clones. In addition, the presence and cover of all plant species was recorded within each quadrat. Field sites in NY were extensively surveyed for their plant communities in 2009 and 2010.

In order to quantify species evenness in these quadrats, the authors calculated Shannon equitability, E_H, as:

$$E_H = H / H_{max} = -\sum_{i=1}^{s}(p_i \ln p_i) / \ln S$$

Where H is the Shannon's diversity index, S is the total number of species in the community, and p_i is the proportion of S made up of the *i*th species.

In order to determine whether native or introduced *P. australis* abundance (cover) had a significant effect on quadrat plant species richness, evenness, height, or litter depth, the team conducted linear multiple regressions with site and *P. australis* cover within site as fixed effects.

Across sites, 51 species were associated with native *P. australis* (richness=17-22 spp./site) and 80 with non-native *P. australis* (richness=14-36 spp./site) (Table 5). Within sites, there was a significant negative correlation between non-native *P. australis* cover and both species richness and evenness (Figure 4). Meanwhile, there was no relationship between native *P. australis* cover and richness or evenness (Figure 4), although native *P. australis americanus* never reached the same high abundance as introduced *P. australis* (Figures 4, 5).

Pharalaris arundinacea was present at the greatest number of sites (N=8). There appears to be a strong competitive relationship between the two introduced grasses (R^2_{adj}=0.22, $F_{1,34}$=10.79, *P*=0.0024, Figure 6), but not with the native *P. australis* cover (R^2_{adj}=0.06, $F_{1,54}$=3.53, *P*=0.0656, data not shown).

Table 5. Species occurrence at the 11 long-term monitoring sites (2009-present). Sites are as follows: 1 = Rochester; 2 = Syracuse; 3 = Bear Swamp; 4 = Utica 51; 5 = Utica 79; 6 = Lakeview; 7 = Cowell Pond; 8 = Carncross; 9 = Martens Marsh; 10 = Eagle Point native; 11 = Eagle Point introduced

	Field Sites										
Plant Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Acer rubrum			x								
Alnus incana ssp. *rugosa*	x		x								
Andropogon gerardii	x										
Apios americana	x	x		x							
Apocynum cannabinum	x							x			
Arctium lappa						x					
Artemisia vulgaris					x						
Asclepias incarnata										x	x
Atriplex patula		x									
Bidens frondosa		x									
Calamagrostis canadensis		x	x					x	x		
Carex lacustris			x	x	x	x					
Carex leptalea			x								
Carex rostra									x	x	x
Carex stricta			x								
Chamaedaphne calyculata			x								
Cirsium arvense	x	x		x	x			x	x	x	
Convolvulus spp.		x				x	x	x		x	
Cornus racemosa	x	x	x								
Cornus rugosa	x			x					x		
Cynanchum rossicum											x

Daucus carota	x	x			x						
Dryopteris spp.			x								
Echinocystis lobata				x							
Epilobium glandulosum	x										
Epilobium hirsutum		x									
Equisetum arvense		x				x	x		x		
Equisetum laevigatum	x										
Eupatorium maculatum		x						x	x	x	
Eupatorium perfoliatum	x										
Euthamia caroliniana					x						
Euthamia graminifolia	x	x			x			x			
Fragaria virginiana	x										
Fraxinus americana				x							
Galium spp.	x	x		x		x	x	x			
Geum aleppicum										x	
Geum canadense		x									
Hypochaeris radicata		x									
Impatiens capensis	x			x		x	x	x		x	x
Juncus spp.	x										
Juncus tenuis	x										
Lathyrus palustris						x					
Leersia oryzoides	x										
Linaria vulgaris					x	x					
Lonicera spp.		x									
Lotus corniculatus	x										
Lycopus americanus	x	x					x	x			
Lycopus uniflorus		x					x				
Lysimachia nummularia		x									
Lythrum salicaria		x		x	x		x	x	x	x	x
Mentha arvensis		x				x					
Mentha piperita	x										
Myrica gale			x								
Onoclea sensibilis								x	x		
Osmunda cinnamomea		x	x	x		x					x
Panicium virgatum	x										
Parthenocissus quinquefolia		x		x		x				x	
Persicaria amphibia				x		x	x				
Persicaria punctata											x
Persicaria sagittatum										x	
Persicaria virginiana	x						x				
Phalaris arundinacea	x			x	x	x	x	x	x	x	x
Photinia melanocarpa			x								
Phragmites australis (Intro)	x	x		x	x		x		x		x
Phragmites australis americanus			x			x		x		x	
Plantago major		x									
Potentilla fructicosa			x								
Potentilla palustris							x				
Ribes spp.				x							
Rudbeckia hirta	x										
Rumex spp.		x									
Salix spp.	x					x					x
Scirpoides holoschoenus	x										

Scirpus ancistrochaetus						x					
Scutellaria galericulata						x					
Sium suave							x				
Solanum dulcamara		x				x	x	x		x	x
Solidago canadensis	x	x		x	x	x					
Solidago grandifolia	x										
Solidago juncea					x						
Solidago puberula								x			
Sparganium eurycarpum							x				
Spartina alterniflora								x			
Spartina pectinata									x		
Sphagnum spp.			x								
Spirea alba var *latifolia*			x								
Stachys palustris									x	x	x
Symphyotrichum lanceolatum		x							x		
Symphyotrichum lateriflorum		x				x					
Symphyotrichum patens				x							
Symphyotrichum puniceum		x									
Symplocarpus foetidus			x		x						
Teucrium canadense								x			
Tovara virginiana	x										
Toxicodendron radicans		x		x							
Typha angustifolia			x								
Typha lat x ang						x	x	x			x
Typha latifolia	x	x			x						
Urtica dioica										x	
Verbascum thaspis		x									
Verbena hastata				x							
Viburnum dentatum		x		x					x		
Vicia villosa	x										
Viola spp.	x										
Vitis riparia		x						x	x		

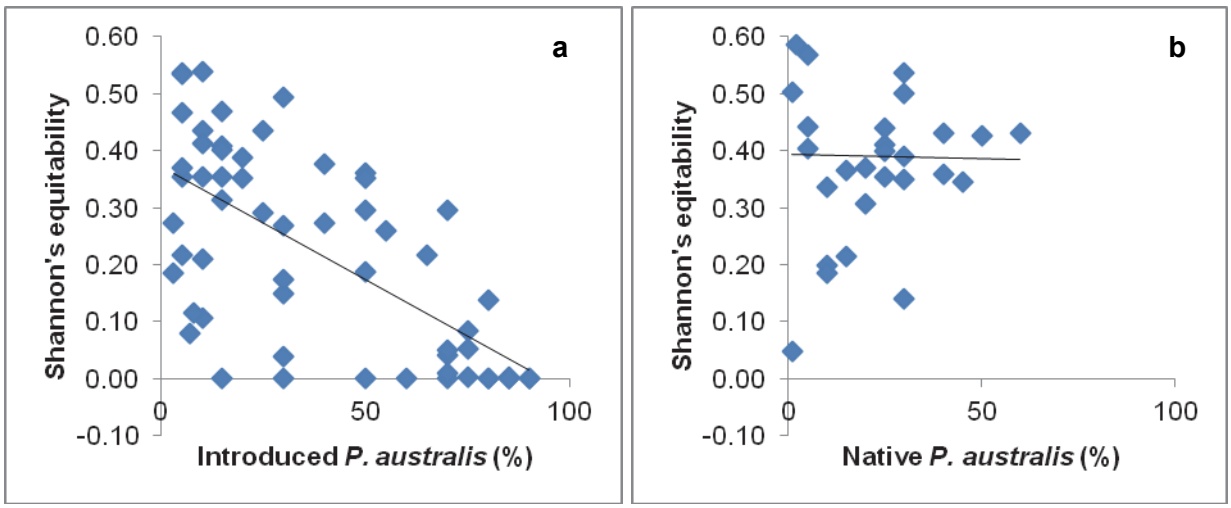

Figure 4. The relationship between cover (%) of introduced (a) and native (b) *Phragmites* and plant community evenness (Shannon's equitability, E_H). Data taken in August 2009 from 15-18 long-term monitoring quadrats per site.

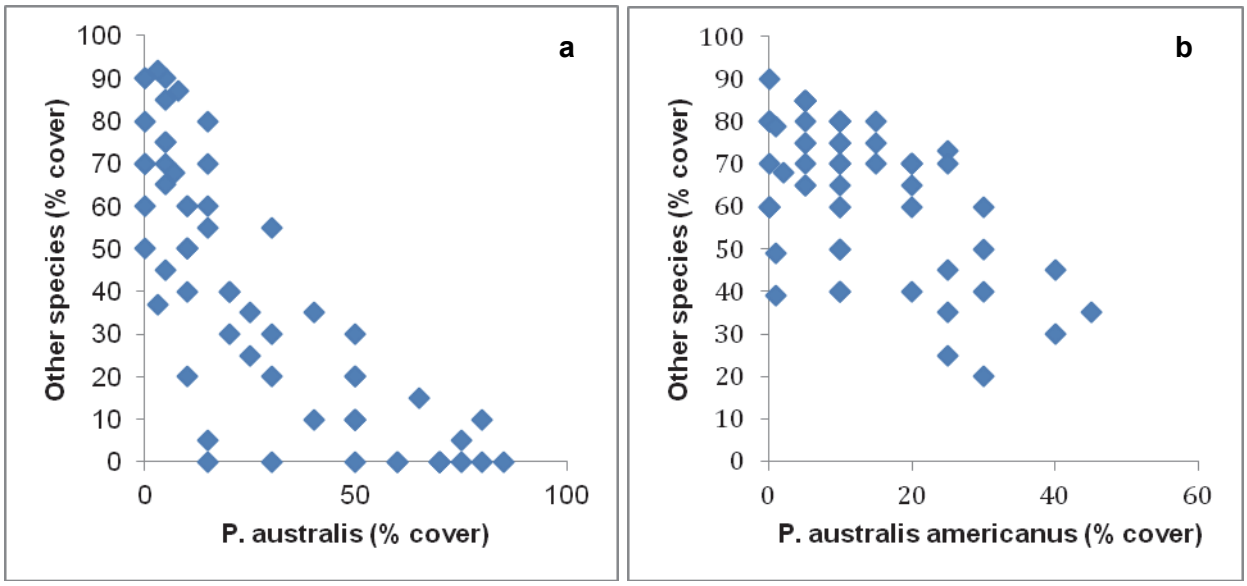

Figure 5. Cover (%) of non-*Phragmites* plant species as a function of introduced (a) and native (b) *Phragmites* cover (%). Data taken in August 2009 from 15-18 long-term monitoring quadrats per site (N= 4 native and 7 introduced populations, Table 4).

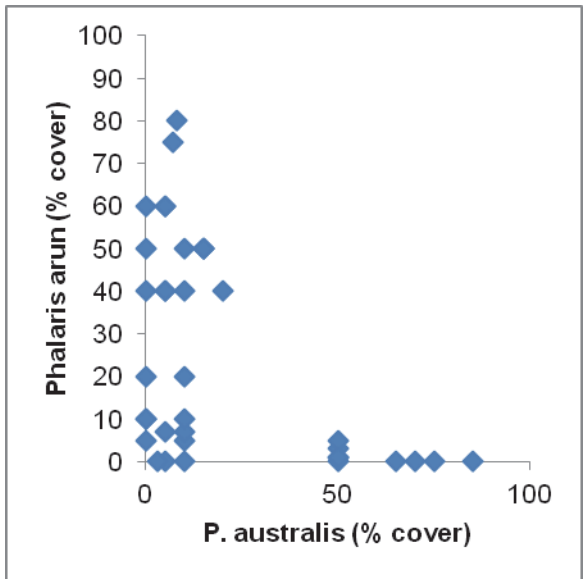

Figure 6. The relationship between percent cover of *Phalaris arundinacea* cover (%) as a function of non-native *Phragmites cover (%)*. Data taken in August 2009 at 7 sites with 15-18 long-term monitoring quadrats/site.

These are preliminary assessments extracted from the first years of Laura Martin's PhD work. The authors will attempt to showcase additional work from the common garden and field monitoring to assess the extent and impact of clonal expansion on the interaction between native and introduced *Phragmites*. The results of the common garden and field monitoring work will

also illustrate the effect of clonal expansion of either genotype on associated wetland plant communities. The initial impression is clearly that a diversity measure — such as Shannon's — index is not an appropriate measure for *Phragmites* impact. Despite the great reduction in cover of other species associated with increasing *Phragmites* cover, the overall effect on species diversity is minimal. This is a misrepresentation of the true invasion effect. The authors have a number of manuscripts in various stages of development and anticipate submitting these in the next year. Due to the loss of some personnel, the team has a backlog of data analyses and recovering this lost ground will be a top priority over the next year.

Objective 5: Assess the extent of hybridization between native and introduced genotypes

Hybridization between native and introduced *P. australis* genotypes has been discussed as a possibility by researchers for a long time. Recently, the possibility of such events has received increased attention with the report that such hybrids can be created/forced in the laboratory (Meyerson et al. 2008). However, until recently, the existence of hybrids in the field has not been reported, despite some significant sampling efforts. The authors now have reliable genetic evidence for a hybrid occurrence in the field at the Montezuma National Wildlife Refuge. The location of this hybrid is in the vicinity of two "parental" clones that grow close to the visitor center. Further searches in the larger Montezuma wetlands complex have revealed the existence of further "morphologically suspicious" individuals. The existence of hybrids complicates not only the development of biological control, but also the management of the species using "traditional" means. Furthermore, some hybrids that Laura Meyerson was able to create appear to indicate hyper-invasiveness (increased biomass production and clonal expansion; L. Meyerson, (unpublished data). The authors have "teamed" with Kristin Saltonstall as a contractor on this project and developed a field collection protocol. Initial sampling was conducted in the Montezuma wetlands complex and the authors have also teamed with New York Department of Environmental Conservation and US Fish and Wildlife Service personnel to survey for stands of "suspicious" clones or individuals (those displaying morphological characteristics of both native and introduced genotypes). Bernd Blossey spent a full day training personnel in the recognition of native and introduced genotypes and the potential hybrids.

Unfortunately, the analysis of the material has been delayed due to problems with obtaining import permits to Panama, where Kristin Saltonstall is currently located. While the team has been finally able to send material with personal courier, at present, it is not possible to report on any progress. First results, however, should be forthcoming. Field visits and characterization of morphology seem to indicate that hybrids may not be as common as previously thought, but only genetic testing will be able to validate this observation.

Outlook. The team continues to make substantial progress in the work program. The 2012 field season will be used to propagate and prepare for a big push in testing the sensitive North American *P. australis americanus* genotypes in Switzerland, using the field release techniques reported on earlier at more sites in 2013. With an additional spring of host specificity testing at URI in 2013, the authors should be near completion of all required tests for two of the four control agents under consideration at the present time. The authors will begin drafting a TAG petition during the winter months.

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